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Substitution of a TATA Box from a Herpes Simplex Virus Late Gene in the Viral Thymidine Kinase Promoter Alters ICP4 Inducibility but Not Temporal Expression

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The role of *cis*-acting promoter elements associated with herpes simplex virus type 1 (HSV-1) early and late genes was evaluated during productive infection with regard to activation of gene expression by the HSV-1 transactivator ICP4 and control of temporal regulation. A set of recombinant viruses was constructed such that expression of an HSV-1 early gene, thymidine kinase (tk), was placed under the control of either the tk TATA box or the TATA box from the late gene, glycoprotein C (gC), in the presence or absence of the upstream Sp1 and CCAAT sites normally found in the tk promoter. The presence of Sp1 sites in the promoter or replacement of the tk TATA box with the gC TATA box resulted in a decreased activation of tk mRNA expression by ICP4. Substitution of the A+T-rich region from the gC TATA box in the context of the remainder of the surrounding tk sequences resulted in a promoter that bound recombinant TATA-binding protein (TBP) better at lower concentrations than the wild-type tk promoter did. These results indicate that tk promoters that are better able to utilize TBP are less responsive to ICP4 activation and suggest that activation by ICP4 involves the general transcription factors that interact with TBP or TBP itself. Additionally, all of the viruses expressed tk at early times postinfection, indicating that *cis*-acting promoter elements that control the level of expression of HSV-1 early and late genes do not determine temporal regulation.

Herpes simplex virus type 1 (HSV-1) genes can be roughly categorized into three classes on the basis of their time of expression during productive infection (18). Immediate-early (IE) genes are expressed shortly after infection, and their transcription does not require prior protein synthesis (18, 19). Early genes are the next class expressed; their synthesis requires the activity of at least one IE protein (19). Among the products of early genes are the DNA replication enzymes. Finally, late genes are expressed to maximum levels following viral DNA synthesis (18, 19). Late genes encode predominantly the structural proteins of the virion. Expression of the early and late genes is absolutely dependent on the presence of functional IE proteins and, in particular, the IE protein ICP4 (11, 42).

ICP4 is a 170-kDa phosphoprotein that is required for activating transcription from most HSV-1 genes during viral infection (11, 42, 58). Mutational analyses have identified domains of the ICP4 molecule that are associated with its transcription-activating function (9, 10, 38, 39, 48); however, the mechanism by which ICP4 transactivates HSV-1 early and late gene promoters remains unclear. ICP4 binds specifically to diverse sequences present in many HSV-1 early and late genes (14, 22, 27, 34, 36, 54); however, the ability of ICP4 to bind to DNA has not been clearly shown to contribute to transactivation (2, 22, 40, 46, 47, 49, 54, 55).

The viral thymidine kinase (tk) promoter is transactivated by ICP4 (6, 8, 35). It is transcribed by RNA polymerase II and contains a CCAAT box and two Sp1 sites upstream of a TATA box (24, 33). Studies of this promoter have found that only the *cis* sequences that interact with cellular transcription factors are required for expression of tk during viral infection; no induction-specific sequences have been identified (3, 5, 12). A more recent study directly evaluated the role of these *cis* sites in ICP4 induction of the tk promoter and found that induction by ICP4 was not dependent on the integrity of these *cis* sequences (21). Efficient induction by ICP4 could be observed in the presence of only the tk TATA box. Therefore, the induction of HSV-1 genes by ICP4 may be mediated through TFIID, the other general cellular transcription factors (TFIIA, TFIIB, and TFIIE/F), or RNA polymerase II. TFIID is composed of TATA-binding protein (TBP) and a number of TBP-associated factors that often mediate interactions with activating proteins (11a). Biochemical studies on the IE transactivator of pseudorabies virus, which shows sequence conservation with ICP4 (4, 57), have shown that IE stabilizes the interaction of TFIID with an inducible promoter (1). Therefore, promoters that possess TATA boxes with a greater affinity for TFIID or TBP may not be affected by the activity or presence of ICP4 as much as are TATA boxes that have a lower affinity for these factors. In this study, the effects of ICP4 mediated induction of two different TATA box sequences with different affinities for TBP were evaluated to test this hypothesis.

HSV-1 late gene promoters differ from early gene promoters in that the *cis*-acting sequences 5′ to the TATA box, such as Sp1 or CCAAT sites, that are prevalent in early promoters (53) are usually not present in late promoters. In general, prior analyses of late gene promoters have indicated that DNA sequences 5′ to the TATA box are not required for induction or for regulation as a late gene (14, 16, 17, 23, 45). Late gene promoters also differ from early promoters by their inability to be maximally expressed until viral DNA replication occurs (18). However, a tk promoter lacking the

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upstream CCAAT and Sp1 sites has been shown to be expressed as an early gene (21). This promoter is similar to HSV-1 late gene promoters in that the only identified *cis*-acting sequence present is the TATA box; nevertheless, it was not expressed as a late gene. This result may be due to differences in the sequence of the TATA boxes. Previous work has suggested that functional differences between early and late gene TATA boxes exist (16).

In this study, a set of chimeric promoters that combined the identified *cis* elements of the early tk promoter and of the late gC promoter was constructed and each was subsequently recombined in place of the wild-type tk promoter in an ICP4-deficient virus, *n*12 (10). This permitted evaluation of tk expression under the control of either the early gene tk TATA box or the late gene gC TATA box in the presence or absence of the upstream Sp1 and CCAAT sites found in the tk promoter. By recombining these promoters into the tk locus of *n*12 (10), expression directed by each promoter could be monitored both in the absence of ICP4, by infecting Vero cells, and in the presence of ICP4, by infecting E5 cells. E5 cells are Vero cells that have been transformed with the ICP4 gene and that, on HSV-1 infection, produce levels of ICP4 that can complement ICP4-deficient viruses (7, 9). Thus the ability of ICP4 to induce each of these promoters can be examined. In addition, the kinetics of tk expression as a function of each promoter can be monitored by infecting E5 cells for various times postinfection.

MATERIALS AND METHODS

Virus and cells. Cells were maintained under conditions previously described (7). The *n*12 viral strain contains a nonsense mutation in both copies of the ICP4 gene at amino acid 251 (10). E5 cells (7, 9) express complementing levels of ICP4 upon HSV-1 infection and were used to propagate *n*12 and *n*12 viruses containing tk promoter mutations. *n*12 d−111/−46 was previously described (21). *n*12 viruses containing other tk promoter mutations are described below.

Plasmids. pLS/*ts*−42/−32 and pLS/*ts*−111/−101 each contain a linker-scanning mutation marked by a *Bam*HI site in the indicated position in the tk promoter (see Fig. 2D). These plasmids have been described previously (5, 33). pd/*ts*−111/−32 was generated by ligation of the 4.4-kb *Bam*HI fragment of pLS/*ts*−111/−101 to the 3.0-kb *Bam*HI fragment of pLS/*ts*−42/−32.

To generate plasmids containing gC TATA boxes in the background of the −42/−32 and −111/−32 mutations, the *Hin*dIII-*Bgl*II (−500 to +54) promoter fragments of pLS/*ts*−42/−32 and pd/*ts*−111/−32 were first cloned into a pUC19 plasmid in which a *Bgl*II linker (New England BioLabs) had been inserted at the *Sma*I site in the polylinker. The resulting plasmids, p−42/−32HB and p−111/−32HB, were then digested with *Bam*HI and with *Mlu*I, which cuts the tk promoter at −13 relative to the start site of tk transcription. Two complementary oligonucleotides were synthesized such that when they were annealed to form double-stranded DNA, the tk sequence between the *Bam*HI site at −42/−32 or at −111/−32 and the *Mlu*I site at −13 was reconstituted, except that the nucleotides making up the tk TATA box were precisely replaced by those encoding the gC TATA box (see Fig. 1B). The sequences of the oligonucleotides are 5′-GATCCGGTTCGTATAAATTGGTGA-3′ and 5′-CGCGTCACCAATTTATACGAACCG-3′. Phosphorylated, double-stranded oligonucleotides were ligated to *Bam*HI-*Mlu*I-digested p−42/−32HB and p−111/−32HB to generate p−42/−32(C)HB and p−111/−32(C)HB, respectively. The presence of the oligonucleotide encoding the gC TATA box was identified by the loss of an *Mse*I site that is encoded within the tk TATA box and was verified by DNA sequencing. The *Hin*dIII-*Bgl*II promoter fragments of these plasmids were then cloned back into the *Hin*dIII-*Bgl*II vector fragment of pLS/*ts*−111/−101 to generate pLS/*ts*−42/−32(C) and pd/*ts*−111/−32(C).

Generation and screening of recombinant viruses. Plasmids LS/*ts*−42/−32 and d/*ts*−111/−32 and their counterparts containing the gC TATA box were linearized at the *Sal*I site in the vector sequence and cotransfected into E5 cells with infectious *n*12 DNA as described previously (15, 21, 37). Selection for the temperature-sensitive thymidine kinase phenotype with acyclovir was performed as described previously (5, 21). Subsequent screening for the tk promoter mutation was performed by Southern blot hybridization (50), using electrophoretically separated digests of DNA from small-plaque cultures and nick-translated probes containing tk promoter sequences.

Isolation of infected-cell RNA and Northern blot analysis. Confluent monolayers of Vero or E5 cells were infected at a multiplicity of infection (MOI) of 10 PFU per cell. Infected-cell RNA was prepared and subjected to Northern (RNA) blot analysis exactly as described previously (21). The probe for tk mRNA levels was a gel-purified, ^{32}P-labeled *Sac*I-*Sma*I fragment from the coding region of the tk gene. This fragment spans +555 to +1217 relative to the start site of tk transcription and was chosen because it does not contain sequences from the overlapping UL24 gene or the adjacent gH gene. Filters were stripped by boiling in distilled water and were probed with a gel-purified ^{32}P-labeled *Bam*HI-*Sal*I fragment internal to the ICP8 gene or with a gel-purified ^{32}P-labeled *Bam*HI-*Sal*I fragment internal to the gC coding region. Probes were labeled by nick translation with *Escherichia coli* DNA polymerase I (30).

Primer extension. Infected-cell RNA (35 μg) was used for primer extension analysis exactly as described previously (21).

Southern blot analysis of replicated viral DNA. Approximately 5×10^5 Vero or E5 cells were infected with *n*12 at a MOI of 10 PFU per cell in the presence or absence of 0.3 mg of phosphonoacetic acid (PAA) per ml. Samples were harvested at 1, 6, or 12 h postinfection and lysed in 0.5% sodium dodecyl sulfate (SDS)–0.2 mg of proteinase K per ml for 4 h at 37°C. Each sample was phenol-chloroform extracted repeatedly, chloroform extracted, ethanol precipitated, and resuspended in 0.1 ml of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The samples were then treated with 0.02 mg of RNase A for 1.5 h at 37°C and were phenol-chloroform extracted, ethanol precipitated, and resuspended in distilled H_2O. The A_{260} of each sample was measured, and 3 μg of each infected-cell DNA was digested with *Bam*HI, separated by electrophoresis on a 0.9% agarose gel, and transferred to nitrocellulose (50). Blots were probed with a gel-isolated, ^{32}P-labeled, *Sac*I-*Sma*I (+555 to +1217) DNA fragment internal to the tk coding sequence. Densitometic measurements of appropriately exposed autoradiograms were made with a Hoefer GS300 densitometer, and the data were processed by using Hoefer software for the Macintosh computer.

Purification of human TBP expressed in bacteria. The human TBP expression plasmid pETHIID and *E. coli* BL21 were generous gifts of Arnold Berk and have been described previously (25). pETHIID-transformed BL21 cells (2 liters) were grown in 2× YT medium (44)–0.4% glucose in the presence of 50 μg of ampicillin per ml at 37°C until the A_{600}

reached approximately 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM, and the cells were grown for a further 3 h at 30°C. The cells were pelleted, resuspended in cold TBS (140 mM NaCl, 5 mM KCl, 25 mM Tricine [pH 7.4], 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), and repelleted. All of the following procedures were performed at 4°C. The cells were resuspended in buffer D (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of antipain per ml) containing 0.3 M KCl. After the total volume had been measured, an appropriate volume of 2 M KCl was added to bring the final concentration of KCl to 0.3 M. The cells were sonicated on ice four times for 30 s each, lysozyme was added to a final concentration of 0.05 mg/ml, and the cells were incubated on ice for 15 min. They were sonicated again on ice four times for 30 s each, and the lysate was centrifuged for 15 min at 15,000 rpm. The supernatant was drawn off and put into cold microcentrifuge tubes, which were then centrifuged for 5 min at 13,000 rpm. Supernatants were pooled and applied to a DEAE-Sephacel column preequilibrated with buffer D containing 0.3 M KCl.

The flowthrough was collected, diluted 1:1 with buffer D, and applied at a rate of 0.8 ml/h to a heparin-Sepharose column preequilibrated with buffer D plus 0.15 M KCl. Bound protein was eluted with a 0.15 to 1.0 M KCl gradient. Fractions were assayed by gel shift with a 29-bp double stranded oligonucleotide encoding the adenovirus E1B TATA box, as described previously (25), and by immunoreactivity to a mouse anti-TBP antibody (generously provided by Robert Roeder, Rockefeller University). Relevant fractions were pooled, concentrated by Centricon 30 microconcentrators (Amicon), and applied at a rate of 1 ml/min to a fast protein liquid chromatography (FPLC) Mono Q column equilibrated with buffer D plus 0.1 M KCl. TFIID immunoreactive material was present in the flowthrough; this was applied at a rate of 1 ml/min to an FPLC Mono S column equilibrated with buffer D plus 0.1 M KCl. Bound proteins were eluted with a continuous 0.1 to 1.0 M KCl gradient. Immunoreactive fractions were frozen in liquid nitrogen and stored at −80°C.

Gel shift assays. Complementary 29-bp oligonucleotides encoding the tk and gC TATA boxes were annealed and end labeled with [α-^{32}P]ATP (New England Nuclear) and T4 polynucleotide kinase (New England BioLabs) as described previously (44). The DNA sequence of the top strand of the tk oligonucleotide is GATCCCACTTCGCATATTAAGGT GACGCG, and the DNA sequence of the top strand of the gC oligonucleotide is GATCGGCCCGGGTATAAATTC CGGAAGGG. The indicated amounts of bacterially expressed human TBP were incubated with 50 pg of probe and 10 μg of poly(dG-dC)-poly(dG-dC) per ml in the binding buffer described by Kao et al. (25). The total reaction volume was 10 μl. Following incubation at room temperature for 30 min, 1 μl of 0.025% bromophenol blue was added. Samples were separated by electrophoresis on a 5% polyacrylamide–0.5× TBE (44) gel at 100 V, vacuum dried, and exposed to film.

DNase I assays. Plasmids −42/−32 HB, −42/−32(C)HB, LS/*ts*−111/−101, and LS/*ts*−29/−18 were digested with *Bgl*II, treated with calf intestinal phosphatase (Boehringer), end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (44), digested with *Eco*RI, and separated on an 8% polyacrylamide gel. The liberated promoter fragment spans from −77 to +54 and was prepared as previously described (22). Probe (0.5 ng) was incubated with bacterially expressed human TBP and 50 ng of poly(dG-dC)-poly(dG-dC) in the binding buffer described by Kao et al. (25). After a 30-min incubation at room temperature, an equal volume of 10 mM $MgCl_2$–5 mM $CaCl_2$ was added and the samples were treated with DNase I (Worthington) for 1 min at room temperature. Reactions were stopped with 20 μl of STOP buffer (100 mM Tris-HCl [pH 7.5], 2% SDS, 0.05 mg of tRNA per ml, 40 mM EDTA, 0.2 mg of proteinase K per ml) and incubated at 37°C for 15 min. The samples were then brought to 100 μl with distilled H_2O, phenol extracted, ethanol precipitated, rinsed with 70% ethanol, and resuspended in loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). After being heated to 95°C, the samples were separated by electrophoresis on an 8% denaturing polyacrylamide gel. Following electrophoresis, the gels were fixed in 10% acetic acid–10% methanol, dried, and exposed to film.

RESULTS

To facilitate precise substitution of the TATA box from the tk gene with the TATA box from the gC gene, we used a plasmid containing a linker-scanning mutation between nucleotides −42 and −32 (5, 33). This mutation alters the nucleotides between −42 and −32 to create a *Bam*HI site. The effect of this mutation has been evaluated extensively in transient expression assays and in the context of viral infection, and in no instance has this alteration been shown to affect tk expression (5, 12, 13, 24, 33). By digesting this plasmid with *Bam*HI and *Mlu*I, which cleaves the tk promoter just 3′ to the TATA box, a short sequence containing the tk TATA box was removed. A double-stranded oligonucleotide designed to restore the tk sequence excised, except for replacing the tk TATA box with the gC TATA box, was cloned into this plasmid as well as into a similarly digested plasmid lacking promoter sequences from −111 to −32. This eliminates the tk upstream Sp1 and CCAAT sites (see Materials and Methods and Fig. 1B). Because the tk TATA box encodes an *Mse*I site and the gC TATA box does not, clones were screened by restriction digestion for the loss of an *Mse*I site. Positive clones were then sequenced to verify the substitution.

The recombinant tk promoters are shown schematically in Fig. 1A. LS−42/−32 contains the linker-scanning mutation between −42 and −32 and possesses all the tk *cis*-acting sequences: a TATA box, a CCAAT box, and two Sp1 sites. LS−42/−32(C) contains the tk upstream sites (Sp1 and CCAAT boxes) but possesses the TATA box from the late gene gC in place of the tk TATA box. d−111/−32(C) is a tk promoter under the control of just the tk TATA box, and d−111/−32(C) is a tk promoter under the control of just the tk TATA box. These promoters were incorporated into the tk locus of the ICP4-deficient virus, *n*12, which possesses a nonsense mutation at amino acid 251 in both copies of the ICP4 gene. The *n*12 ICP4 molecule is severely truncated and has no measurable ICP4 function (10). Promoter activity can therefore be measured in the context of viral infection as a function of the promoter alteration in the presence and absence of ICP4.

Southern blot hybridization was performed on selected virus progeny to detect incorporation of the recombinant tk promoters. The probe used was a gel-purified, nick-translated fragment of the tk promoter spanning −500 to +54. Figure 2A shows a Southern blot of a *Bam*HI digest of recombinant viral DNA that demonstrates the incorporation of the *Bam*HI site marking the LS−42/−32 and d−111/−32

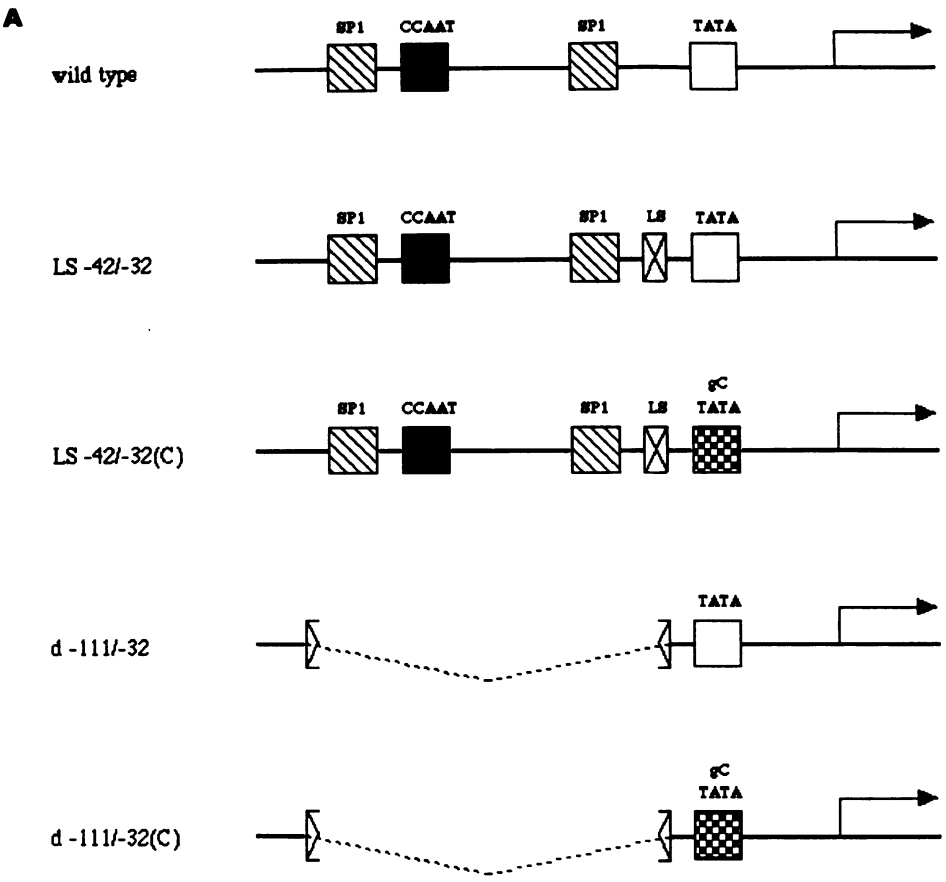

FIG. 1. Schematic diagrams of recombinant tk promoters and sequence of the TATA boxes present in each. (A) Recombinant tk promoters. LS−42/−32 contains the linker-scanning (LS) mutation between −42 and −32 (33) and possesses all of the tk *cis*-acting sequences (a TATA box, a CCAAT box, and two Sp1 sites). LS−42/−32(C) contains the tk upstream sites in conjunction with the late gene gC TATA box in place of the tk TATA box. d−111/−32 is a tk promoter lacking the upstream Sp1 and CCAAT sites, and d−111/−32(C) also lacks the upstream Sp1 and CCAAT sites but is under the control of the gC TATA box. (B) DNA sequence between −40 and −11 of the wild-type and mutant tk promoters. Construction of these promoters is described in Materials and Methods.

mutations. The presence of the LS−42/−32 mutation cleaves the 3.6-kb *Bam*P fragment into 2.9- and 0.69-kb fragments. As the tk promoters of the viruses containing the d−111/−32 mutation are 70 nucleotides shorter, the corresponding *Bam*HI fragment is 0.62 kb. Figure 2B is a *Sac*I-*Pvu*II digest of viral DNA that also confirms the deletion in the viruses containing the d−111/−32 mutation. Figure 2C is an *Mse*I digest of viral DNA. Replacement of the tk TATA

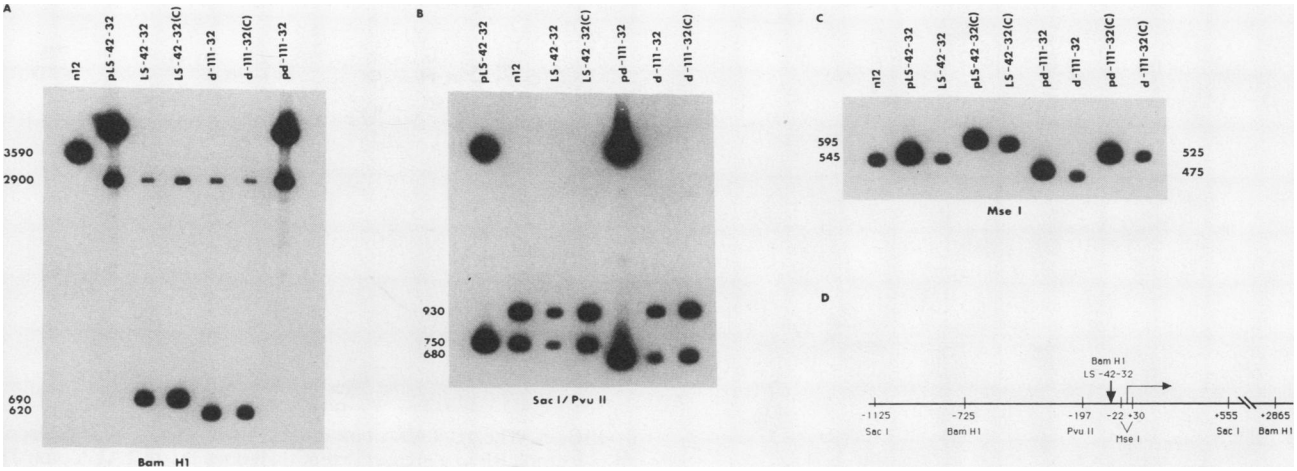

FIG. 2. Southern blots of *n*12 viruses containing mutant tk promoters. A gel-purified, nick-translated tk promoter fragment spanning −500 to +54 of the tk promoter was used in Southern blot hybridizations to detect incorporation of recombinant tk promoters in the *n*12 virus. (A) A *Bam*HI digest of viral DNA demonstrates the incorporation of the *Bam*HI sites marking the LS−42/−32 and d−111/−32 mutations. The 3.6-kb fragment containing the tk gene is cleaved into two smaller fragments in the recombinant viruses. (B) A *Sac*I-*Pvu*II double digest demonstrates the presence of the deletion between −111 and −32 in *n*12 d−111/−32 and d−111/−32(C), as the tk promoter fragment from these viruses is 70 bp smaller than the corresponding fragment from the LS−42/−32 and LS−42/−32(C) viruses. (C) An *Mse*I digest demonstrates the substitution of the gC TATA box for the tk TATA box. The tk TATA box encodes an *Mse*I site. Replacement of the tk TATA box with a gC TATA box eliminates this site and results in a band 50 nucleotides larger. (D) Map of the relevant restriction sites for these Southern blots.

box with the gC TATA box eliminates an *Mse*I site; therefore, the *Mse*I promoter fragment generated by digestion of the LS−42/−32(C) and d−111/−32(C) viruses are 50 bp larger than the corresponding *Mse*I promoter fragments from LS−42/−32 and d−111/−32. Figure 2D is a schematic representation of the tk promoter indicating the location of restriction enzyme sites relevant to the Southern blots.

Induction of mutant tk promoters by ICP4. To show that tk expression from the *n*12 virus containing the LS−42/−32 mutation was quantitatively the same as from the wild type in the presence of ICP4 as well as in the absence of ICP4, we measured mRNA levels by Northern blot analysis following infection of Vero and E5 cells for 6 h in the presence of 0.3 mg of PAA (a DNA replication inhibitor) per ml. Infection of Vero and E5 cells allowed evaluation of tk expression in the absence and presence of ICP4, respectively. Lanes 3 to 6 of Fig. 3 indicate that on infection, *n*12 viruses containing either a wild-type tk promoter (lanes 3 and 4) or the LS−42/−32 promoter (lanes 5 and 6) generated equivalent levels of tk mRNA in the presence of ICP4 and also generated equivalent levels of tk mRNA in the absence of ICP4. Consequently, the LS−42/−32 mutation does not alter tk expression. Also shown in Fig. 3 is a comparison of tk mRNA levels generated by infection of Vero and E5 cells with *n*12 d−111/−32 (lanes 7 and 8) and *n*12 d−111/−46 (lanes 9 and 10). *n*12 d−111/−46 deletes from −111 to −46 of the tk promoter, which includes the upstream CCAAT and Sp1 sites and was previously described (21). *n*12 d−111/−32 also deletes the tk upstream sites but extends the deletion to −32, just 5′ of the TATA box. Both viruses express similar levels of tk mRNA that are 15 to 20% of the wild-type level, in agreement with previous findings (21). Detectable expression was not observed in the absence of both the upstream *cis* sites and ICP4 (lanes 7 and 9). This lack of expression may be due to the greatly increased requirement for the Sp1 sites in the tk promoter in the absence of ICP4, as was previously proposed (21).

Figure 4 shows a Northern blot that compares the mRNA levels for each of the viruses diagrammed in Fig. 1A. Vero and E5 cells were infected with the indicated viruses in the presence of 0.3 mg of PAA per ml, and RNA was harvested at 6 h postinfection. The *n*12 viruses with the wild-type tk promoter and the LS−42/−32 promoter generated equivalent tk mRNA levels when uninduced by ICP4 (lanes 1 and 3). tk expression was induced 33- and 36-fold, respectively, by the presence of ICP4 (lanes 2 and 4). Infection with *n*12 LS−42/−32(C), which substitutes the tk TATA box with the gC TATA box in a promoter containing the upstream Sp1 sites and CCAAT box, did not greatly affect the level of

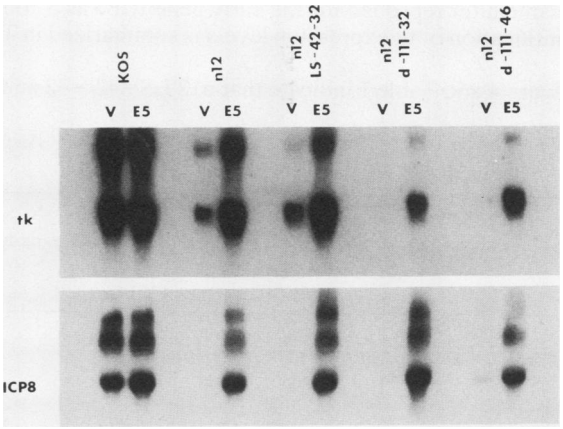

FIG. 3. The LS−42/−32 mutation does not alter tk expression in the absence or presence of ICP4. Vero and E5 cells were infected at an MOI of 10 PFU per cell with the indicated recombinant viruses for 6 h in the presence of 0.3 mg of PAA per ml. tk mRNA levels were assayed by Northern blot. The filter was stripped by boiling in distilled H_2O for 20 min and was reprobed for ICP8, another early gene, as a control. ICP8 mRNA levels are shown in the lower panel.

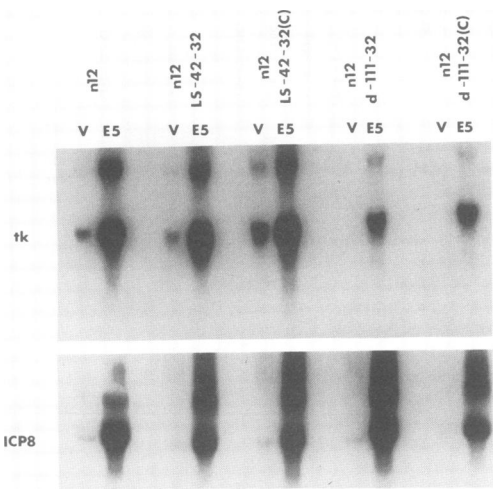

FIG. 4. Substitution of the tk TATA box with the gC TATA box increases basal expression of the tk promoter but has minimal effect on ICP4-induced expression. Vero and E5 cells were infected at an MOI of 10 PFU per cell with the indicated recombinant viruses for 6 h in the presence of 0.3 mg of PAA per ml. tk mRNA levels were assayed by Northern blot. The filter was subsequently stripped and reprobed for ICP8 as a control. ICP8 mRNA levels are shown in the lower panel.

ICP4-induced expression (lane 6). However, in the absence of ICP4 (lane 5), the presence of the gC TATA box increased tk mRNA levels approximately sevenfold. Consistent with previous observations (21) (Fig. 3), expression was too low to be detected when the virus *n*12 d−111/−32 was used to infect Vero cells (lane 7). Replacement of the tk TATA box with the gC TATA box in this context also resulted in undetectable levels of tk mRNA (lane 9). Thus, the presence of the late gene TATA box did not alter the requirement for the upstream binding sites in the absence of ICP4. In the presence of ICP4, the tk and gC TATA boxes directed reduced but similar levels of expression in the absence of upstream binding sites, again indicating that the presence of the late gene TATA box did not significantly affect the level of ICP4-induced expression. The lower panel of Fig. 4 shows the same filter reprobed for the early gene ICP8 as a control. Quantification of tk expression levels is summarized in Table 1.

Figure 4 and Table 1 indicate that n12 LS−42/−32 and n12 LS−42/−32(C), viruses that contain tk promoters that possess the upstream Sp1 and CCAAT sites, show induction levels by ICP4 that are reduced relative to those in their counterpart viruses, *n*12 d−111/−32 and *n*12 d−111/−32(C), which do not contain the upstream sites. The results also show a reduction in the ability of ICP4 to induce tk when the tk TATA box is replaced by the gC TATA box. This results from an increase in the level of uninduced expression (Fig. 4, lane 5). The simplest explanation for the increase seen in Vero cells is that the gC TATA box has a greater affinity for the cellular transcription factor TBP, which binds to the TATA box.

TABLE 1. Relative expression of tk mRNA

Virus	Relative expression of tk mRNA[a] in:		Induction ratio[b]
	Vero cells (−ICP4)	E5 cells (+ICP4)	
*n*12	1	33	33
*n*12 LS−42/−32	1	36	36
*n*12 LS−42/−32(C)	6.5	37	5.7
*n*12 d−111/−32	<0.02[c]	5.5	>275
*n*12 d−111/−32(C)	<0.02[c]	7.6	>380

[a] The values given are in arbitrary units based on densitometric scanning of appropriately exposed autoradiograms. tk mRNA levels generated by infection of Vero cells with *n*12 were normalized to 1; all other tk mRNA levels are expressed relative to this value.

[b] The induction ratio is defined as the ratio of the level of expression in E5 cells to that in Vero cells.

[c] Lower limit of detectability.

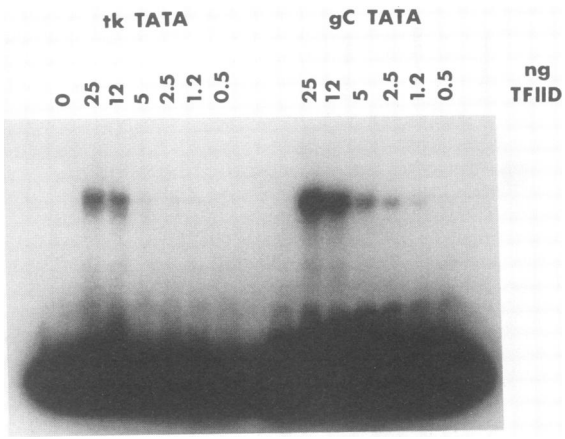

FIG. 5. The gC TATA box interacts with the cellular transcription factor TBP to a greater extent than does the tk TATA box. The indicated quantities of purified human rTFIID (TBP) expressed in bacteria were assayed by gel shift with ^{32}P-labeled double-stranded oligonucleotides encoding either the tk or the gC TATA box.

To test this possibility, we used bacterially produced TBP in a gel shift assay with ^{32}P-labeled oligonucleotides encoding the gC or tk TATA box. Figure 5 shows the result of this experiment. Serial twofold dilutions of TBP were tested for the ability to shift both the tk and gC oligonucleotide probes. The intensity of the shifted band at 25 ng of TBP was much greater with the gC TATA oligonucleotide than with the tk oligonucleotide, and the existence of a shifted band could still be seen when 1.2 ng of TBP was used with the gC oligonucleotide, whereas 5 ng of TBP was needed to produce a similar shift with the tk oligonucleotide. These results indicate that the gC TATA box interacted with TBP to a greater extent than did the tk TATA box.

To confirm these results and to show that interactions between the DNA and TBP were occurring at the TATA box, we performed DNase I footprinting. ^{32}P-end-labeled promoter fragments spanning from −77 to +54 were isolated from plasmids containing tk promoters with the indicated TATA boxes. After incubation with TBP, DNase I was added to the binding reaction and the labeled DNA was analyzed to identify protected regions on the promoter. Figure 6A compares the interaction of TBP with the wild-type tk promoter fragment and with the corresponding fragment from a plasmid containing a linker-scanning mutation in the tk TATA box (LS−29/−18 [33]). The highest levels of TBP used gave an almost complete footprint over the wild-type TATA box that extended from nucleotide −35, 7 nucleotides from the 5′ edge of the TATA box, to the 3′ edge of the TATA box. It also appears that at the highest concentrations of TBP used, nucleotides from the 3′ edge of the TATA box to approximately nucleotide +6 were par-

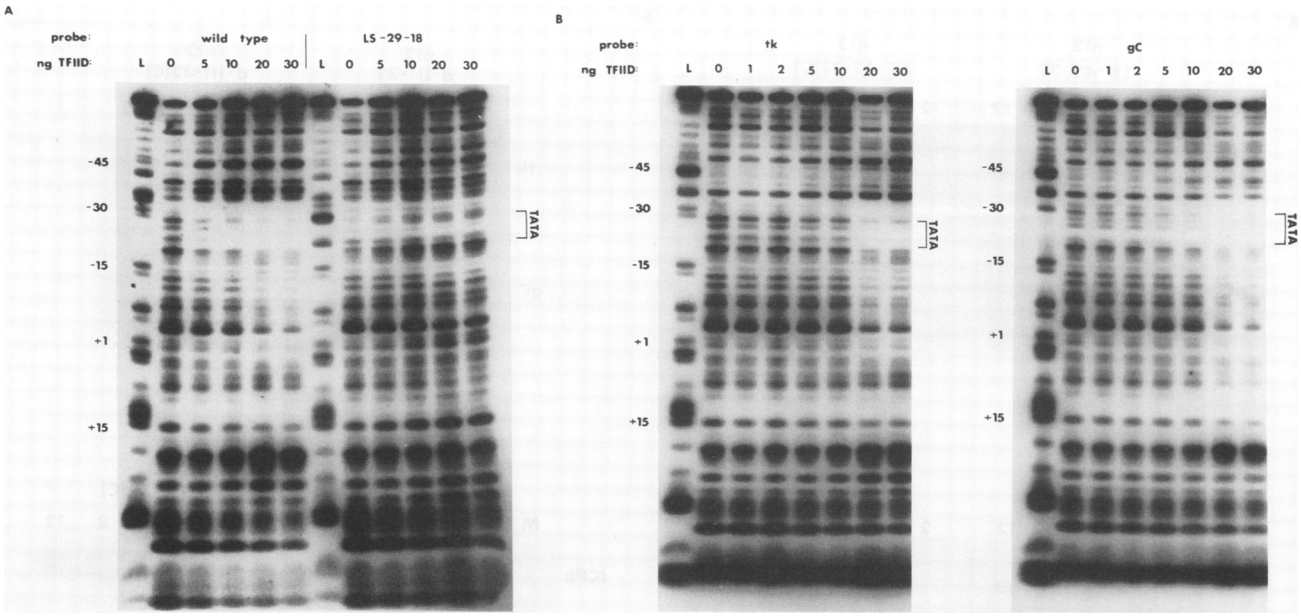

FIG. 6. DNase I footprinting of rTFIID-promoter interactions. (A) End-labeled promoter fragments spanning −77 to +54 of the wild-type tk promoter and a tk promoter containing a mutated TATA box (LS−29/−18) were incubated with the indicated amount of rTFIID (TBP), treated with DNase I, and analyzed by electrophoresis. (B) End-labeled promoter fragments spanning −77 to +54 of the plasmids encoding the LS−42/−32 and LS−42/−32(C) tk promoters were incubated with the indicated amount of rTFIID (TBP), treated with DNase I, and analyzed by electrophoresis.

tially protected. A duplicate experiment performed with the LS−29/−18 probe resulted in no footprint, indicating the specificity of the interaction between TBP and the DNA. In Fig. 6B, promoter fragments from plasmids LS−42/−32 and LS−42/−32(C), which contain the gC and tk TATA boxes, respectively, in the context of the tk promoter, are compared. Levels of TBP that generated a complete footprint over the gC TATA box gave only a partial footprint over the tk TATA box. This result reflects the ability of the gC TATA box to interact with TBP to a greater extent than the tk TATA box does and is in agreement with the results presented by the gel shift assay (Fig. 5). In addition, the 5′ and 3′ limits of the footprint were identical for both the gC and tk TATA boxes.

Kinetics of expression of mutant tk promoters. The results in Fig. 4 show that all of the promoters directed the expression of tk at 6 h postinfection in the presence of a DNA synthesis inhibitor. This indicates that each of these promoters, including the d−111/−32(C) promoter, which is driven by the late gC TATA box in the absence of the upstream Sp1 and CCAAT sites, could be expressed at early times and did not require DNA synthesis. Thus the gC TATA box was not sufficient to confer the properties of a late gene to the tk promoter. To confirm that no DNA synthesis was occurring under the conditions used here, we determined the extent of DNA replication in the presence of 0.3 mg of PAA per ml. Vero and E5 cells were infected with the *n*12 virus for 1, 6, and 12 h postinfection in the presence or absence of PAA. Viral DNA was isolated from the infected cells and digested with *Bam*HI, and a Southern blot was performed to analyze amplification of sequences in the tk gene as a representation of the amount of viral DNA replication (Fig. 7). The results show that in Vero cells, in the absence of ICP4, the level of tk DNA was minimal and equivalent for 1, 6, and 12 h postinfection, whether PAA was present or not. Infection of E5 cells in the absence of PAA indicated that tk DNA was being amplified. In the presence of PAA, no increase in the level of tk DNA was seen. Therefore, 0.3 mg of PAA per ml was sufficient to inhibit DNA replication.

Although each of the promoters tested was expressed at early times, tk mRNA levels were monitored over time to determine whether the presence of the gC TATA box could extend tk expression to late times. E5 cells were infected with *n*12 LS−42/−32 and *n*12 LS−42/−32(C), and RNA was isolated at 2, 4, 6, 8, and 12 h postinfection. tk mRNA levels were examined by Northern blot (Fig. 8A, top) and primer extension (Fig. 8A, bottom) analyses. For both viruses, a peak of tk expression was reached at 6 h postinfection and

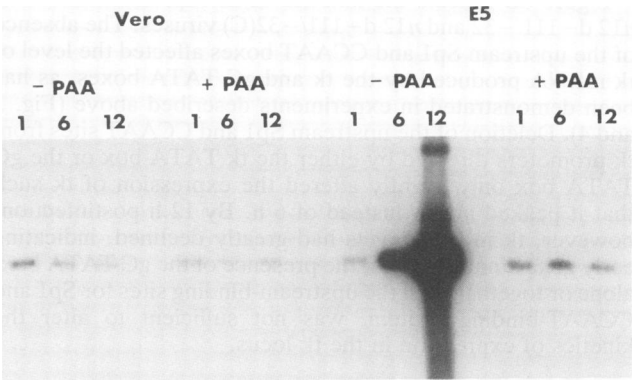

FIG. 7. DNA replication is inhibited by 0.3 mg of PAA per ml. As a control for the experiments that require inhibited DNA synthesis, Vero and E5 cells were infected at an MOI of 10 PFU per cell with the *n*12 virus for 1, 6, and 12 h in the presence or absence of 0.3 mg of PAA per ml. Viral DNA was isolated from infected cells and digested with *Bam*HI, and a Southern blot was performed to analyze replication of the tk gene.

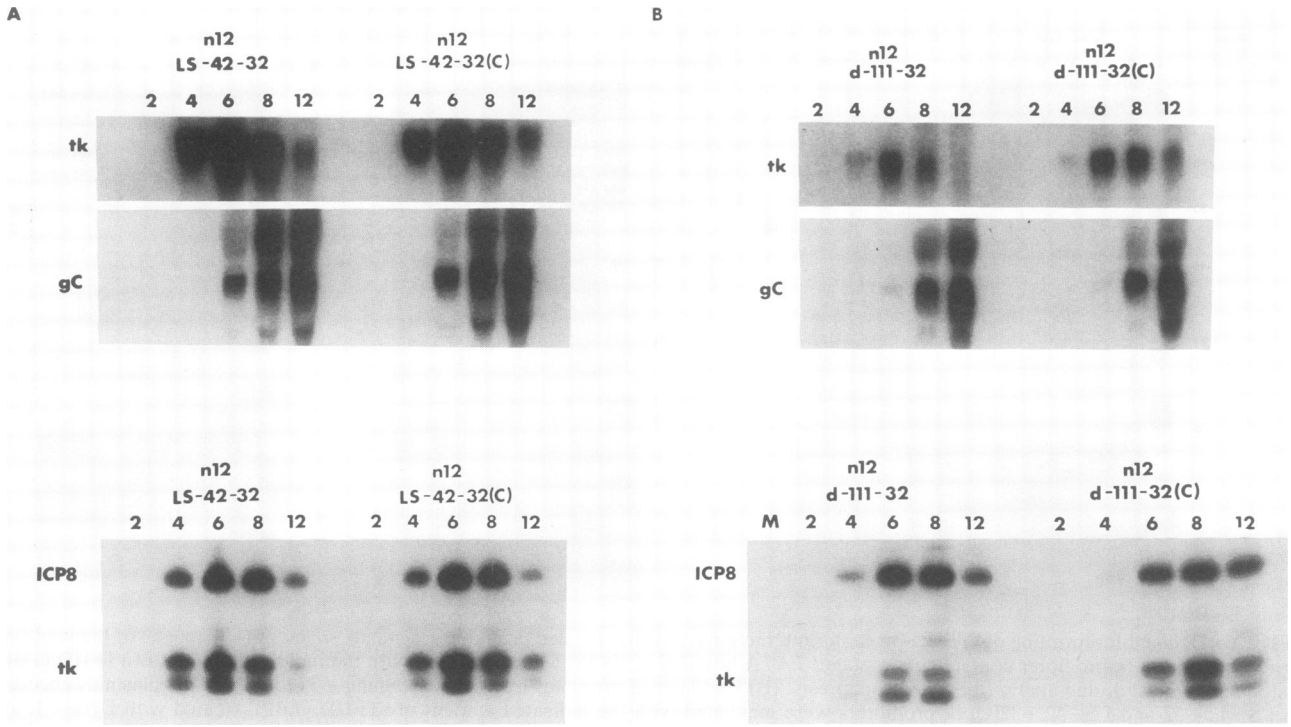

FIG. 8. The recombinant tk promoters are expressed with the kinetics of an early gene. E5 cells were infected at an MOI of 10 PFU per cell with the indicated recombinant viruses for 2, 4, 6, 8, and 12 h. (A) tk mRNA levels from cells infected with *n*12 LS−42/−32 and *n*12 LS−42/−32(C) were assayed by Northern blot (top panel) and primer extension assays (bottom panel). Following hybridization to the tk probe, nitrocellulose filters were stripped and reprobed for gC, a late gene. In the primer extension assays, ICP8 mRNA levels were simultaneously measured as a control. (B) tk mRNA levels from cells infected with *n*12 d−111/−32 and n12 d−111/−32(C) were monitored by Northern blot and by primer extension assays. Following hybridization to the tk probe, nitrocellulose filters were stripped and reprobed for gC. In the primer extension assays, ICP8 mRNA levels were simultaneously measured as a control.

mRNA levels were significantly decreased by 12 h postinfection. This contrasts with the expression pattern of the late gC gene, for which the Northern blot shown in Fig. 8A was reprobed. gC mRNA was detectable at 6 h postinfection and was present at higher levels at 12 h postinfection. A similar pattern for tk expression was observed by primer extension. Expression levels of ICP8, also an early gene, were simultaneously monitored as a control in this assay.

Figure 8B reflects the kinetics of tk expression from the *n*12 d−111/−32 and *n*12 d−111/−32(C) viruses. The absence of the upstream Sp1 and CCAAT boxes affected the level of tk mRNA produced by the tk and gC TATA boxes, as has been demonstrated in experiments described above (Fig. 3 and 4). Deletion of the upstream Sp1 and CCAAT sites from tk promoters directed by either the tk TATA box or the gC TATA box only slightly altered the expression of tk such that it peaked at 8 h instead of 6 h. By 12 h postinfection, however, tk mRNA levels had greatly declined, indicating early gene kinetics. Thus, the presence of the gC TATA box, alone or together with the upstream-binding sites for Sp1 and CCAAT-binding protein, was not sufficient to alter the kinetics of expression in the tk locus.

DISCUSSION

The role of specific *cis*-acting sequences associated with HSV early (tk) and late (gC) genes during productive infection was evaluated with regard to the inducibility of expression by the HSV *trans*-acting protein ICP4. Recombinant promoters directing expression of the tk gene were constructed and recombined into the virus to evaluate tk expression under the control of either the tk TATA box or the gC TATA box, alone or in conjunction with upstream binding sites for the cellular transcription factors Sp1 and CCAAT-binding protein. Since the promoters were recombined into an ICP4-deficient virus, the effect of ICP4 could be directly evaluated by infecting Vero cells and E5 cells, which produce complementing levels of ICP4 on infection by HSV. Induction by ICP4, then, can be defined as the ratio of expression measured in E5 cells (with ICP4) to that in Vero cells (without ICP4).

ICP4 induction of the mutant promoters. The results of this study and those previously presented (21) indicate that the level of induction of a promoter by ICP4 can be affected by the presence of specific binding sites for cellular transcription factors. Adding the Sp1 and CCAAT sites to a promoter containing either the tk or gC TATA box resulted in a greater increase in uninduced than in induced expression. Therefore, promoters in this study containing only the TATA boxes were induced by ICP4 to a greater extent than were those that also contained the upstream sites. In addition, the fold induction by ICP4 was influenced by the apparent affinity of the TATA box for TBP. Substitution of the tk TATA box with the gC TATA box in the presence of the Sp1 and CCAAT sites resulted in a 5- to 10-fold increase in uninduced expression but had little effect in the presence of ICP4. Thus, the level of induction of this promoter by ICP4 was decreased relative to the wild type. The increase in RNA levels as a result of the gC TATA box in the absence of ICP4 was correlated with an increased ability of TBP to bind

to the gC TATA box relative to the tk TATA box. The gel shift assay (Fig. 5) and the DNase I footprinting assay (Fig. 6B) indicate that equal levels of specific interaction between TBP and the promoter were achieved with approximately fourfold less TBP when the promoter contained the gC TATA box than when it contained the tk TATA box.

Despite the large variation in relative strengths of these promoters under uninduced conditions, the presence of ICP4 still caused induction of gene expression such that significant levels of tk mRNA were produced. Thus, no specific *cis*-acting sequence element is required for induction by ICP4. This is in agreement with previous findings (3, 5, 21). Furthermore, the relative strength of the promoter under uninduced conditions does not necessarily correlate with its relative strength in the presence of ICP4. Thali et al. (56) have proposed that the pseudorabies virus IE protein, which shows structural similarity to the ICP4 molecule (4, 57), induces only "sub-optimally utilized" promoters, such that IE function is limited to, or specific for, promoters that are not maximally transcribed, such as those found in PRV genes. The data presented here are in agreement with this proposal, in that the weakest promoters under uninduced conditions, which are under the control of only the tk or gC TATA box, gave the highest induction levels and the strongest promoter under uninduced conditions, which contains the tk upstream sites hooked to the gC TATA box, had the lowest induction level (Table 1; Fig. 4). Thus, an increase in the affinity of the TATA box for TBP or the presence of Sp1 sites in the promoter reduced the induction of tk expression by ICP4. Since Sp1 has been shown to activate expression via a coactivator that functions through TBP (41, 43), these results suggest that a promoter that is better able to utilize TBP is less responsive to induction by ICP4. Therefore, ICP4, either alone or in conjunction with other factors, may promote or stabilize the interaction of TBP with the promoter, as has been shown for the IE protein of pseudorabies virus (1) and the Zta transactivator of Epstein-Barr virus (29). Given that previous results have shown that a mutation in the CCAAT box of the tk promoter had no effect on ICP4 induction levels (21), the effect of the factor binding to the CCAAT box may be independent of TBP. The simplest mechanism by which ICP4 may promote or stabilize TFIID interactions at the promoter is by direct interaction between ICP4 and TBP or TFIID. The ability of TBP to interact in vitro with the viral *trans*-activators VP16 of HSV (52), Zta of Epstein-Barr virus (29), and E1A of adenovirus (20, 28) has been documented. In addition, ICP4 may stabilize TBP-promoter interactions by not only interacting with TBP, but also interacting with other transcription factors that interact with TBP, such as TBP-associated factors, TFIIB, or TFIIA. Preliminary studies suggest that this may be the case (5a).

Kinetics of expression. The data presented in this study demonstrate that the presence of upstream binding sites for cellular transcription factors does not determine the temporal pattern of tk mRNA accumulation resulting from transcription at the natural tk locus. Moreover, substitution of T+A-rich core comprising the TATA box of a true late gene had no effect on the temporal pattern of tk expression from this locus in the presence or absence of the upstream factors.

Previous studies analyzing HSV late gene promoters have generally indicated that sequences from just 5′ of the TATA box to the translation initiation site are sufficient to direct late gene expression (14, 16, 17, 23, 45). Homa et al. (16) postulated that a specific 15-bp sequence including the TATA box mediated expression of the gC gene at late times, because substitution of this TATA sequence with sequences containing the tk TATA box or random A+T-rich sequences resulted in no expression and deletion of the 5′ untranslated leader sequences affected only the level of mRNA accumulation and not the time of expression. Explanations for the difference between their results and those presented in this study include the possibility that sequences flanking the TATA box contribute to the temporal pattern of expression and that the position of the promoter in the genome may also contribute to the determination of kinetic class. It may be interesting to examine the temporal pattern of expression from a given promoter element at its natural site and at several ectopic sites in the viral genome. Results published by Steffy and Weir (51) showed that TATA boxes from IE, early, and late HSV genes were essentially interchangeable in a recombinant late gene promoter expressed from the viral genome, further implying that structural features other than the TATA box determine late gene expression.

The results suggest that the *cis*-acting binding sites for cellular transcription factors act predominantly to determine the level of expression and not when a promoter is activated. However, fusion of Sp1 and CCAAT sites to late gene promoters has been shown to result in expression at both early and late times (16, 32). If these sites, as our data indicate, only increase expression levels, then perhaps the presence of such sites in a late promoter increases the strength of the promoter such that the restriction that prevents expression of late genes until the onset of DNA replication is overcome. The data presented here suggest that this restriction is not due to any of the defined sequences in early or late gene promoters that bind to cellular transcription factors. It has been proposed that sequences present in the 5′ untranslated leader sequences of late genes contribute to late gene expression (26, 32). Such sequences could bind to a cellular or viral protein that represses transcription by preventing the formation of an active transcription initiation complex. Alternately, a *cis* sequence could affect the competence of a promoter for expression by affecting the secondary structure of the template, as has been proposed previously (31). However, other reports have shown that elimination of the gC leader sequences did not alter temporal regulation (59). It remains to be determined whether late gene leader sequences contribute to expression at late times because of an effect on transcription initiation or an increase in mRNA stability. The potential role of transacting factors and *cis*-acting sites operating in the leaders or at the start sites of late genes is an area for future studies.

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